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Human Umbilical Cord Blood Myeloid Progenitor Cells Are Relatively Chemoresistant: A Potential Model for Autologous Transplantations in HIV-Infected Newborns

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Vertical transmission from mother to child occurs in 15–39% of women infected with the human immunodeficiency virus (HIV). Stem cell transplantation has recently been suggested as a potential therapy for patients with HIV infection. We have examined the possible advantages of human cord blood (HUCB) stem cells over bone marrow (BM) stem cells in the treatment of HIV-infected newborns. HUCB myeloid progenitors were found to be statistically more resistant to interferon-α (IFN-α), cytarabine (ARA-C), and eilatin than BM myeloid progenitor cells grown with IL-3 ($P < 0.05$). HUCB treated with IFN-α, ARA-C, and eilatin demonstrated a significantly higher capacity for self-renewal manifested by delta assay following 7 days in liquid culture. We, therefore, suggest that HUCB purged by anti-HIV drugs may be a source for autologous transplantation in HIV-infected newborns. Am. J. Hematol. 56:161–167, 1997.

Key words: human umbilical cord blood (HUCB); interferon-α (IFN-α); ARA-C; eilatin; colony-forming units; liquid culture; self-renewal; human progenitor cells (HPC)

INTRODUCTION

Vertical transmission of the human immunodeficiency virus (HIV) from mother to child occurs in 15–39% of HIV-infected women and is the main cause of infection in children. About 25% of the infected children develop AIDS in the first year of life [1,2].

Zidovudine therapy during pregnancy or delivery significantly reduces both vertical transmission and AIDS development [3]. Cytarabine and cytarabine derivatives have been shown to be effective in the treatment of AIDS and AIDS-related complications [4–7]. Interferon-α (IFN-α) is a cytokine that resulted in the stimulation and activation of CD4 positive cells, and elevation of the CD4/CD8 ratio in AIDS patients [8–10]. It has also been successful in the treatment of AIDS-related Kaposi's sarcoma [11,12]. Moreover, IFN-α has been used in combination with zidovudine, hematopoietic growth factors, and BMT in the treatment of AIDS [12,13].

The rationale underlying BMT for HIV-infected patients is that the main reservoir of cells containing HIV consists of lymphocytes, (primarily CD4+), macrophages, myeloid cells, Langerhans dendritic cells, B lymphocytes transformed by EBV, and microglial cells [14–22], which are precisely the cells destroyed by the massive chemoradiotherapy used prior to BMT. These cells are reconstituted from donor-derived human progenitor cells (HPC), after BMT [23]. Allogeneic and syngeneic BMT are, therefore, potential treatments for patients with AIDS [24–26]. Nevertheless, to date experience with allogeneic BMT in HIV-infected individuals has been rather limited [23]. The main problem in the cases studied is that the HIV infection was not completely eradicated.

An intriguing possibility is the application of autologous BMT to HIV-positive patients. There are two major routes of research. The first takes advantage of the fact that HPC have been demonstrated to be resistant to HIV

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[24]. Using polymerase chain reaction (PCR) technology, the CD34 cells do not appear to contain HIV gene sequences, which raises the possibility that virus-negative HPC may be obtained from HIV-infected individuals [26–27]. Hence, the positive selection of CD34 cells is currently being investigated as a means to reconstitute HIV-positive patients following auto BMT. An alternative approach is to develop purging techniques to destroy HIV-positive cells in a manner analogous to that used to remove malignant cells in patients undergoing autologous BMT for malignant diseases. Our work relates to the second alternative.

Human umbilical cord blood (HUCB) as a potential source of HPC has recently been shown to be successful in hematopoietic reconstitution following myeloablative chemoradiotherapy [28–31]. HUCB is rich in HPC and early progenitors with high repopulating and replating ability [32–36]. The hematopoietic progenitors in HUCB seem to be more primitive than their counterparts in BM or peripheral blood: The colonies in agar or methyl cellulose are bigger and the multipotential colony-forming units (CFU-Mix) and highly proliferative potential colony-forming cells (HPP-CFC) are more numerous. Compared to adult BM stem cells, the cells survive longer and expand with different growth factors in long-term cultures [32–38]. Integration of retroviruses or adeno-associated viruses is more effective in HUCB-enriched progenitor cells than in adult BM [39–44]. Since primitive quiescent stem cells in BM are relatively resistant to chemotherapy [45–48], we hypothesized that HUCB progenitor cells may be even more chemoresistant than BM progenitor cells. In this study, we studied the sensitivity of HUCB progenitor cells to IFN-α and ARA-C, both known to have anti-HIV activity, and eilatin which has so far proved to be an inhibitor of proliferation of progenitor cell in myeloid diseases, and demonstrated their relative resistance, compared to BM HPC. No such difference was demonstrated with AZT. We therefore speculated that it should be possible to treat HUCB from HIV-infected newborns with anti-HIV agents including ARA-C and INT-α, and later perform autologous HUCB stem cell transplantation followed by zidovudine therapy.

MATERIALS AND METHODS

Human BM and CB Samples

Human BM marrow cells were obtained from three consenting individuals without any hematologic disorder. The cells were anticoagulated with heparin, washed, and suspended in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Grand Island, NY).

Samples of placenta and attached cord scheduled for discard after delivery of the infant, were obtained from three newborns according to Hadassah University Hospital guidelines. Delivery room personnel were instructed to leave a hemostat in place on the placental side of the cord after transection. Within 20 min after delivery, the cord blood was collected by elevating the placenta and releasing the hemostat, allowing the blood to flow by gravity into heparinized tubes containing preservative-free grade 1 sodium heparin (Sigma, St. Louis, MO) at a final concentration of at least 20 U/ml.

Light-density (<1.077 g/cm^3), non-adherent cells were obtained by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation and attachment of mononuclear cells to plastic tissue dishes in IMDM with 7.5% fetal bovine serum (FBS). The non-adherent cells were removed after 60 min incubation at 37°C, as previously described [34].

Drugs

ARA-C (Upjohn, Kalamazoo, MI) was dissolved in 0.9% NaCL solution immediately prior to use. Lypholized recombinant IFN-α (Roche Products, Welwyn, UK) was dissolved in IMDM immediately prior to use. Eilatin (MW356) was isolated and purified from the Red Sea purple Tunicate Eudistoma sp. as described by Shochet et al. [49]. A stock solution of 10^{-3} was prepared in dimethyl sulfoxide (DMSO) (Sigma). The drug was diluted with IMDM before use to the appropriate concentration. Human interleukin-3 (IL-3), stem cell factor (SCF), and granulocyte-macrophage colony stimulating factor (GM-CSF) produced in *Escherichia coli* were received as a generous gift from Genetics Institute, Inc. (Cambridge, MA). FBS was purchased from Hyclone Laboratories, Logan, UT. The mouse monoclonal antibody (MoAb) anti HPCA-2 (CD34) (clone 8G12, IgG1) and the isotype-matched (IgG1) control anti-rota virus MoAb were purchased from Becton Dickenson, Rodemark, Germany. Fluorescin-isothiocyanate conjugated (FITC) goat anti-mouse IgG (GAM IgG) was purchased from Bio-Yeda, Rehovot, Israel.

Exposure of Non-Adherent Mononuclear Cells to IFN-α, ARA-C, Eilatin, and Zidovudine in Agar Cultures

Non-adherent mononuclear BM cells or HUCB were brought to a concentration of 1×10^6 cells/ml in IMDM containing 15% heat-inactivated FBS, glutamin 2 mM, and antibiotics. The cells were exposed to IFN-α (500 and 1,000 U/ml), ARA-C (10^{-10}–10^{-8}M), Eilatin (10^{-8}–10^{-6}M), or zidovudine (10^{-7}–10^{-5}M) for 16 h, after which time the cells were washed twice in phosphate-buffered saline (PBS) and cultured in agar. Cells (1.5×10^5) in 1 ml IMDM containing 15 PBS, 15 U/ml IL-3 (or alternatively 500 pM SCF, 15 U/ml GM-CSF, or a combination of hematopoietic growth factors), and 0.3% agar were added to 35 mm Petri dishes. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO2/

95% atmosphere for 10 days. Colony-forming units granulocyte-macrophages (CFU-GM) containing more than 40 cells were counted on day 10 using an inverted microscope.

Exposure of Non-Adherent Mononuclear Cells to IFN-α, ARA-C, and Eilatin and Delta Assay

Non-adherent mononuclear cells from BM or HUCB were suspended at a concentration of 1×10^6 cells/ml in IMDM containing 15% FBS. IFN-α (1,000 U/ml), ARA-C (10^{-8}M), Eilatin (10^{-6}M), or zidovudine (10^{-5}M) were added and the cells were incubated for 16 h at 37°C in 5% CO2/95% atmosphere. After incubation, the cells were washed twice with PBS, and resuspended in IMDM, and 1.5×10^5 cells were plated in agar. For suspension culture 15×10^5 cells were plated in flat-bottomed 24-well tissue culture plates (Lindboro, Flow). After incubation for 7 days in liquid cultures at 37°C in 5% CO2/95% atmosphere, the cells were collected, counted, and replated in agar culture at a concentration of 15×10^5 cells/mL with 15 U/ml IL-3. Colonies with 40 or more cells were counted on day 10 as described above.

Secondary plating efficiency (delta assay), was calculated as the number of CFU-GM generated after 7 days in liquid cultures relative to the number of colonies generated from day 0 (equivalent to day 10 CFU-GM in agar) from marrow or HUCB cells.

Flow Cytometry

Cells (5×10^5) were incubated with anti-CD34 MoAb to isotype control MoAb at 4°C for 30 min and washed twice with PBS to which 20 μL of FITC-conjugated MoAb was added. The cells were then incubated for an additional 30 min at 4°C, and fluorescence was measured on a FACS 440 fluorocytometer (Becton Dickinson, San Jose, CA).

Statistical Analysis

Univariate comparison of means for statistical difference was performed by unpaired two-tail Student's *t*-test; $P < 0.05$ was considered statistically significant.

RESULTS

Effect of IFN-α on CFU-GM Growth From BM and HUCB

The cloning frequency of non-adherent cells isolated from BM or HUCB grown with IL-3 (15 U/ml) was 229 ± 1.3 and 62 ± 4 CFU-GM, respectively, per 1.5×10^5 cells. Exposure of the cells to IFN-α (500 or 1,000 IU/ml) resulted in a significant inhibition of CFU-GM growth (n = 3, $P < 0.05$). Inhibition was considerably higher for CFU-GM grown from BM than from HUCB (55 ± 5.2% vs. 28 ± 1.3% inhibition, respectively, for 500 U/mL IFN-α and 76 ± 2.1% vs. 50 ± 1.7 for 1,000 U/mL IFN-α) (n = 3, $P < 0.05$) (Fig. 1).

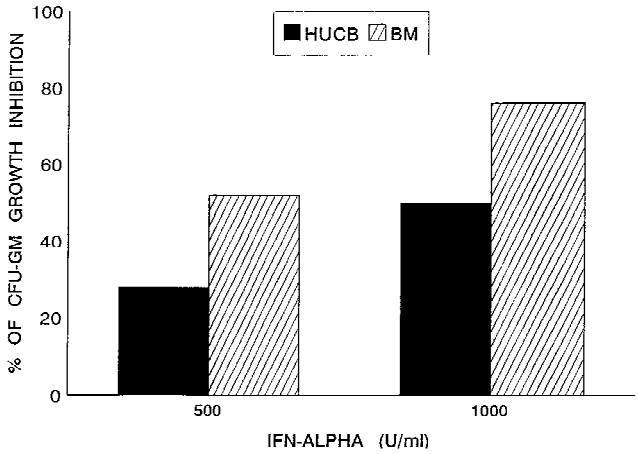

Fig. 1. The effect of increasing concentrations of IFN-α (500 and 1,000 U/ml) on CFU-GM growth from BM and HUCB. Non-adherent cells were obtained following plastic adherence as described under Materials and Methods. Our normal plating efficiency for BM and HUCB in the presence of IL-3 (15 U/ml) is 229 ± 1.3 and 62 ± 4.0 CFU-GM, respectively, per 1.5×10^5 cells. Results are presented as % of CFU-GM growth inhibition compared to controls with no IFN-α (n = 3 ± SE) (*$P < 0.05$).

Effect of ARA-C on CFU-GM Growth From BM and HUCB

Exposure of BM and HUCB cells to ARA-C (10^{-10}M, 10^{-9}M, and 10^{-8}M) resulted in a significant, dose-dependent inhibition of CFU-GM cloning frequencies (n = 3, $P < 0.05$) (Fig. 2). Considerably higher inhibition was noted for CFU-GM grown from BM (30, 62, and 100% inhibition, respectively) than for HUCB (3, 31, and 72% inhibition, respectively) (n = 3, $P < 0.05$) for all evaluated concentrations of ARA-C (Fig. 2).

Effect of Eilatin on CFU-GM Growth From BM and HUCB

Eilatin (10^{-8}M, 10^{-7}M, and 10^{-6}M) significantly inhibited CFU-GM growth in a dose-dependent manner (Fig. 3). Again, significantly higher inhibition was observed for CFU-GM grown from BM (40, 45, and 100% inhibition, respectively) than from HUCB (17, 22, and 50% inhibition, respectively) (n = 3, $P < 0.05$). Maximal inhibition was observed at 10^{-6} Eilatin (Fig. 3).

Effect of Zidovudine on CFU-GM Growth From BM and HUCB

Zidovudine alone significantly inhibited CFU-GM growth in a dose-dependent manner. No notable differences were observed between the inhibitory effect of zidovudine on BM and HUCB (Fig. 4).

Zidovudine in combination with a whole panel of

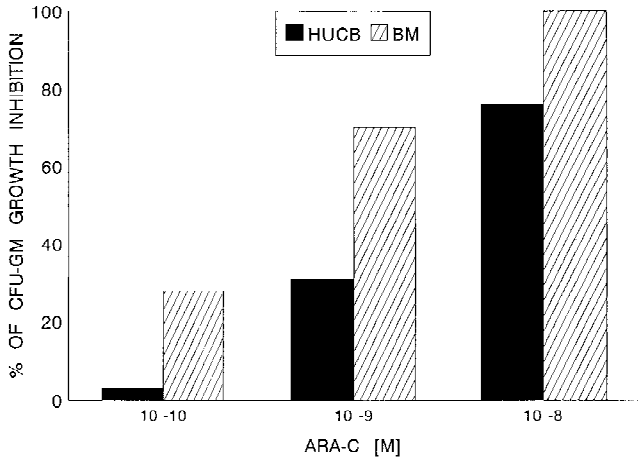

Fig. 2. The effect of increasing concentrations of ARA-C (10^{-10}–10^{-8}M) on CFU-GM growth from BM and HUCB.

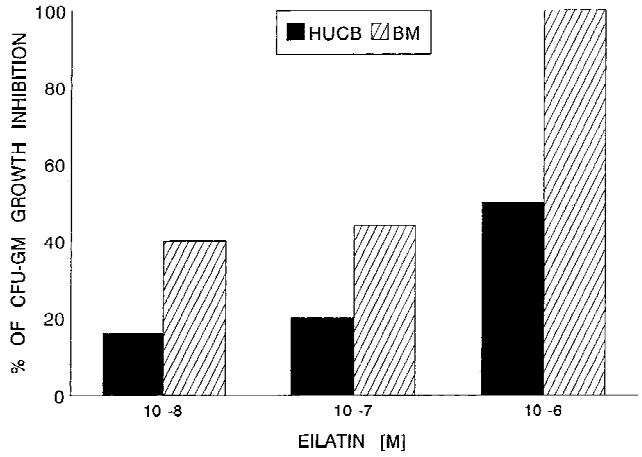

Fig. 3. The effect of increasing concentrations of Eilatin (10^{-8}–10^{-6}) on CFU-GM growth from BM and HUCB.

growth factors including GM-CSF, IL-3, SCF, GM-CSF+SCF, and IL-3+SCF, significantly inhibited both BM and HUCB CFU-GM growth in a dose-dependent manner (Figure 5). Here, too, no difference was observed between the magnitude of inhibition between BM and HUCB.

Effect of IFN-α, ARA-C, and Eilatin on Cell Recovery Following 7 Days Liquid Culture

No significant change was demonstrated in the recovery of cells in BM and HUCB following 7 days of liquid culture as shown in Table I ($2.9 \pm 1 \times 10^6$ colonies in BM vs. $3.07 \pm 0.5 \times 10^6$ colonies in HUCB with no addition of drugs, $2.3 \pm 0.8 \times 10^6$ colonies in BM vs. $2.8 \pm 0.4 \times 10^6$ colonies in HUCB with the addition of IFN-α, $2 \pm 0.9 \times 10^6$ colonies in BM vs. $2.7 \pm 0.6 \times 10^6$ colonies in HUCB with the addition of ARA-C, and $1.9 \pm 0.4 \times 10^6$ in BM vs. $2 \pm 0.3 \times 10^6$ colonies in HUCB with the addition of Eilatin).

CD34, with IFN-α, ARA-C, and Eilatin too, was evaluated in BM and HUCB pre- and post-7-day liquid culture. No significant difference was observed (data not shown).

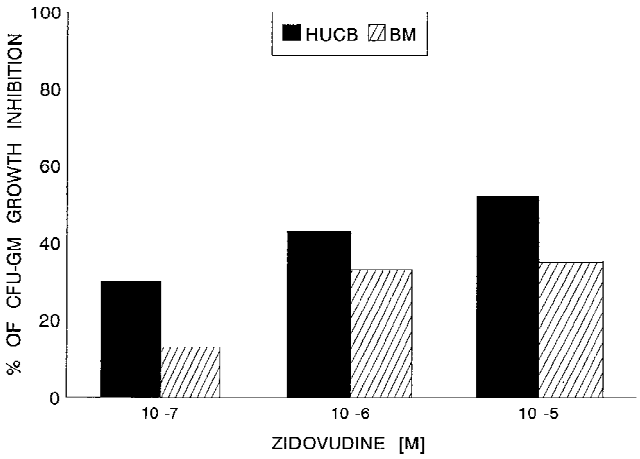

Fig. 4. The effect of increasing concentrations of zidovudine (10^{-7}–10^{-5} on CFU-GM growth from BM and HUCB.

Effect of IFN-α, ARA-C, and Eilatin on Secondary Plating (Delta Assay) of BM and HUCB

Following 7 days in liquid cultures, the number of secondary colonies grown with IL-3 was 2,300 ± 81 for BM and 1,342 ± 47 for HUCB, per 1.5×10^5 cells. Significantly diminished growth of secondary colonies was observed both with IFN-α 1,000 U/ml and with ARA-C 10^{-8}M in BM compared to HUCB (60 vs. 9%) and 30 vs. 11%, respectively) ($n = 3$, $P < 0.05$) (Table I), while with Eilatin 10^{-6}M, the magnitude of inhibition was similar for BM and HUCB (64 vs. 70%, respectively) (Table I).

The secondary plating efficiency (delta assay) following 7 days' liquid cultures was considerably higher for HUCB than for BM (21.6 vs. 10) ($n = 3$, $P < 0.05$) (Table I). This significant difference was sustained following the addition of IFN-α (19.8 vs. 3.98), ARA-C (19.2 vs. 6.8), and Eilatin (6.4 vs. 3.6) ($n = 3$, $P < 0.05$) (Table I).

DISCUSSION

Previous studies have demonstrated that HUCB HPC seem to be more primitive than their counterparts in BM or peripheral blood [32–44]. In the present study, we have demonstrated that the anti-HIV agents IFN-α and ARA-C have a greater effect on BM HPC than HUCB HPC. HUCB myeloid progenitor cells proved relatively resistant to the antiproliferative effect of these agents. This may indicate that the HUCB HPC are less sensitive than BM-derived HPC. The greater sensitivity of colony formation by BM than by HUCB most likely reflects a higher percentage of cycling progenitors in the BM, and

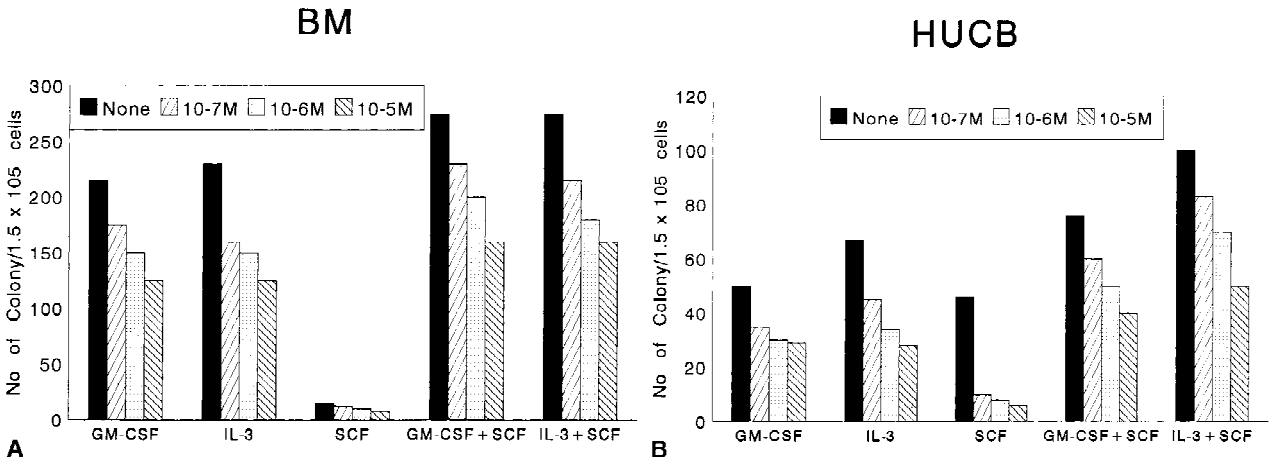

Fig. 5. The effect of increasing concentrations of zidovudine (10^{-7}–10^{-5}) in conjunction with IL-3, GM-CSF, and SCF on CFU-GM from BM (A) and HUCB (B).

TABLE I. Growth of Non-Adherent Mononuclear Cells in Liquid and Semisolid Cultures Following Exposure to IFN-alpha, ARA-C, and Eilatin*

Treatment	No. of primary colonies[a]	% inhibition[b]	No. of colonies recovered (×106)	No. of secondary colonies[c]	% of inhibition	Delta assay[d]
			Bone marrow			
None	229 ± 1.3	0	2.9 ± 1	2,300 ± 81	0	10
IFN-alpha (1,000 U/ml)	55 ± 1.2	76%	2.3 ± 0.8	913 ± 34	60	3.98
ARA-C (10M)	0	100%	2 ± 0.9	1,576 ± 42	30	6.8
Eilatin (10^{-6}M)	0	100%	1.9 ± 0.4	827 ± 31	64	3.6
			Human umbilical cord blood			
None	62 ± 4	10	3.07 ± 0.5	1,342 ± 47	0	21.6
IFN-alpha (1,000 U/ml)	31 ± 1.2	50%	2.8 ± 0.4	1,233 ± 12	9	19.8
ARA-C (10_{-8} M)	17.3 ± 1.1	72%	2.7 ± 0.6	1,195 ± 34	11	19.2
Eilatin (10^{-6}M)	32.8	47%	2 ± 0.3	402 ± 11	70	6.4

*Values represent mean ± SE of experiments performed in duplicate (n = 3).

[a]Non-adherent mononuclear cells (1×10^6/ml) were incubated with the above-mentioned compounds for 16 h. After incubation, the cells were isolated and resuspended in IMDM, and 1.5×10^5 were plated in agar.

[b]Inhibition percentage was calculated as follows: The differences between the number of colonies before and after exposure to the drugs, divided by the number before exposure ×100.

[c]Non-adherent mononuclear cells (1×10^6 cells/mL) were incubated with the above-mentioned compounds for 16 h. The cells were then washed and resuspended in IMDM, and 1.5×10^5 cells/mL were plated in 24-well tissue culture plates. After incubation for 7 days in liquid culture, cells were collected, counted, resuspended in IMDM and 1.5×10^5 cells/ml were replated in agar (secondary colonies).

[d]Secondary plating (delta assay) was defined as secondary colonies in agar devided by number of colonies in day 0 with IL-3.

thus a greater sensitivity to the cytotoxic agents. Eilatin, a novel marine product isolated from the Red Sea purple Tunicate Eudistoma sp, which has previously been shown by us to possess antiproliferative effects against the HPC of chronic myeloid leukemia patients [50], was found to be more effective in inhibiting primary colonies grown in agar than in suspension cultures. This indicates that Eilatin primarily affects the clonogenicity rather than the self-renewal capacity of HPC.

The significantly higher secondary plating (delta assay) that we observed in HUCB compared to BM following incubation with IFN-α, ARA-C, and Eilatin is further evidence of the more primitive nature of HUCB HPC and its higher capacity for self-renewal and secondary colony formation.

Zidovudine is a key drug in the treatment of AIDS patients. We therefore evaluated its inhibitory effect on the growth of BM and HUCB CFU-GM in conjunction with GM-CSF, IL-3, SCF, GM-CSF+SCF, and IL-3+SCF, but failed to demonstrate any notable differences.

Our findings may have important clinical applications for HIV-infected newborns. Extraordinary success has recently been achieved in dramatically reducing the risk of mother-to-child transmission of HIV using prenatal and intrapartum zidovudine [3]. Nevertheless, a high per-

centage of these children will develop AIDS or AIDS related complications including high-grade malignancies [3–7]. The therapeutic options for HIV-infected mothers and children are rather limited, consisting of zidovudine, IFN-α, ARA-C, avoidance of breastfeeding, cleansing of the birth canal, and, possibly in the future, immunization [4–10]. BMT is also a potential treatment for patients with AIDS, including children [13,23–26]. BM HPC have previously been shown to be resistant to HIV. In this study we have demonstrated that HUCB HPC are even more resistant to the antiproliferative agents IFN-α and ARA-C currently used for the treatment of AIDS patients. We therefore, propose a novel therapeutic strategy for HIV-infected mothers and their offspring consisting of: (1) administration of zidovudine to mothers during pregnancy, labor, and delivery; (2) purging the HUCB of the newborn with a combination of IFN-α ARA-C and Eilatin or other even more potent drugs (we are currently performing in vitro experiments to find out whether these drugs or others indeed have the ability to to eradicate HIV infection); (3) performing autologous HUCB stem cell transplantation on the newborn following myeloablative chemotherapy; and (4) zidovudine treatment post-transplant to prevent re-infection by HIV.

We suggest that this novel approach should be tested as it may prolong disease-free survival in the newborn, reduce HIV-related complications including malignancies, and even offer a potential curative treatment for these children.

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